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Arachidonic acid and human bone marrow stromal cells

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Abstract

Human bone marrow stromal cells regulate the growth of marrow hematopoietic progenitors by secreting cytokines. Arachidonic acid (AA) is the fatty acid precursor of prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) that modulate the growth of human bone marrow progenitors. We have investigated the incorporation of AA in human bone marrow stromal cell cultures, their production of PGE_2 and LTB_4 and the effect of AA on their growth. Gas chromatography analysis reveals the presence of AA in the human bone marrow plasma and in bone marrow stromal cell cultures. In stromal cells, [^{3}H]-AA is incorporated into triglycerides and is later delivered into phospholipids. Prelabeling-chase experiments indicate a preferential incorporation of AA into phosphatidylethanolamine and no trafficking of labeled AA between phospholipid species. Bone marrow stromal cells release PGE_2 and LTB_4 in response to phorbol myristic acetate (PMA) (1 μM) and tumor necrosis factor alpha (TNF-α) (10 ng/ml). Exogenous AA (up to 1 μM) has no significant effect on cell growth. In conclusion, human bone marrow stromal cells capt exogenous AA and, thus, may participate to the control of marrow AA concentrations. They may also regulate human marrow hematopoiesis by secreting AA metabolites such as PGE_2 and LTB_4. © 1998 Elsevier Science B.V.

Keywords: Arachidonic acid; Leukotriene B_4; Marrow stromal cell; Proliferation; Prostaglandin E_2

1. Introduction

Arachidonic acid (AA) is a 20-carbon polyunsaturated fatty acid which is released after stimulation from membrane phospholipids and which can be converted to prostaglandins through the cyclooxygenase pathway or to hydroxyeicosatetraenoic acid (HETE) and to leukotrienes through the lipoxygenase pathway [1]. AA metabolites such as prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) modulate human hematopoiesis by acting on the growth of human bone marrow erythroid and myeloid progenitors in vitro [2–5]. Normal hematopoiesis which is located in the bone marrow is a dynamic and interactive process between hematopoietic cells and stromal cells (mostly fibroblast-like cells). Marrow stromal cells are crucial for the long-term survival, proliferation and differentiation processes of hematopoietic stem cells and progenitors. They regulate hematopoiesis by interacting directly (cell-to-cell contact) with hematopoietic cells and/or by secreting regulatory molecules (such as cytokines and growth factors) that modulate hematopoiesis [6].

The presence and the role of AA in the human marrow is poorly documented. In this study we have investigated AA in human bone marrow plasma and marrow stromal cell cultures. We have assessed the

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uptake, the distribution and the trafficking of AA among triglycerides and phospholipids in human bone marrow stomal cells and the effects of AA on their growth. We also investigated their capacity to release PGE_2, 6-keto-$PGF_{1\alpha}$ and LTB_4 in response to phorbol myristic acetate (PMA), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α).

2. Material and methods

2.1. Bone marrow stromal cell cultures

Cultures of human bone marrow stromal cells were established from bone marrow aspirates harvested into heparinized tubes (Vacutainer system, Becton Dickinson, Meylan, France) and considered as normal by morphological analysis of the May Grunwald Giemsa stained smears. The procedure was performed on patients undergoing a myelogram as part of routine procedure. Mononuclear cells were isolated by separation on a Ficoll gradient ($400 \times g$, 20 min), washed two times with HBSS ($400 \times g$, 10 min), and seeded in 75 cm^2 culture flasks in RPMI 1640 supplemented with 20% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in 5% CO_2 in air [7]. After one week, nonadherent cells were removed from culture flasks. Adherent cells were grown to confluence for 3 to 5 weeks with weekly changes of medium and were subcultured after trypsin treatment (0.05% trypsin for 5 min). The cells used in these experiments were at the first passage. As previously reported [8], more than 99.8% of cells were $CD2^-$ and $CD22^-$ indicating the absence of T- and B-cells on the layers and 4% of cells were $CD14^+$ and $CD33^+$ indicating a monocytic lineage.

2.2. Fatty acid analysis

Bone marrow plasma was obtained by centrifugation ($400 \times g$, 15 min) of marrow samples collected by aspiration into heparinized tubes. Bone marrow plasma lipids were then extracted according to Floch et al. [9]. Marrow stromal cells (1×10^6) were plated into 75 cm^2 culture flask with RPMI 1640 with 20% FCS with or without exogenous AA (1 μM). After 5 days of growth, supernatants were removed and adherent cells were washed with HBSS. Cells were scraped using a rubber policeman, and lipids were extracted [9]. Fatty acids from marrow plasma and marrow stromal cell cultures were then methyl transesterified, and the resulting fatty acid methyl esters were separated by gas chromatography [10]. Standards were run to identify the compounds quantified by gas chromatography.

2.3. Incorporation of [^{3}H]-AA in marrow stromal cells

Trypsinized cells (1×10^5) were plated in 6-well plates in 5 ml of RPMI 1640 with 20% FCS and were grown 5 days. Subconfluent cultures (2 to 3×10^5 cells per well) were washed three times with HBSS to eliminate FCS before use. The incorporation of [^{3}H]-AA (Amersham, les Ulis, France) was performed with cells in classical monolayer maintained in 1 ml of RPMI 1640 medium without FCS. [^{3}H]-AA (0.1 μCi; 210 Ci/mmol) was added into the medium for 10 min, 1, 5 and 24 h. At the end of the experiments, supernatants were recovered and acidified to pH 3 (citric acid 3 M). Supernatants and adherent cells were then stored at −20°C until used. In some experiments AA incorporation was investigated with cells grown for 48 h in serum-free medium before labeling. In other experiments AA incorporation was made with 20% FCS in the medium.

For AA remodelling studies, cells were labeled with [^{3}H]-AA for 2 h, washed with HBSS, and incubated in 1 ml of RPMI 1640 without FCS for 2, 5 and 22 h or used immediately. Cells and supernatants were stored as above.

2.4. Lipid extraction

Acidified supernatants were extracted with diethyl ether and ethylacetate [11]. Lipid extracts were evaporated, recovered in 150 μl of diethyl ether, applied to a thin layer chromatography (TLC) plate (Silica gel 60 (20×20 cm, 0.25 mm), Merck) and submitted to TLC. The plate was developed in the organic phase of the mixture: ethyl acetate/isooctane/acetic acid/water (11:5:2:10, v/v/v/v) [11]. Adherent cells were scraped, mixed with 1 ml of 0.1% sodium

dodecyl sulfate in water, and incubated for 30 min at 56°C with 3 ml of chloroform/methanol (2:1, v/v). The chloroformic extract was washed with 5 ml of 0.1 M KCl/methanol/chloroform (96:92:6, v/v/v), and was evaporated [11]. The dry extract was recovered in 150 μl of chloroform and applied to a TLC plate. The plate was developed in the mixture of diethyl ether/hexane/acetic acid (70:30:1; v/v/v). Each lane was divided in areas of 0.5 cm length which were scraped into vials and radioactivity was measured on a Packard liquid scintillation counter. Solutions of monoglycerides (MG), diglycerides (DG), triglycerides (TG), phospholipids (PL) and AA were used as standards and visualized with iodine vapour.

2.5. Analysis of labeled PL

For the separation of the various species of cellular PL, the corresponding areas were scraped from the TLC plates and were extracted with chloroform/methanol (2:1, v/v). Samples were then rechromatographed using a solvent system of chloroform/methanol/acetic acid/water (50:30:8:4, v/v/v/v) [11]. Each lane was divided in areas of 0.5 cm length and processed as above. Solutions of phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylethanolamine (PE) were used as standards and visualized with iodine vapour. In this chromatography, PI and PS migrated to the same area.

To ensure that the labeled compound in cellular PL was authentic AA, labeled PL were submitted to base hydrolysis [12]. The labeled PL were resuspended in 1 ml of 2 M KOH in ethanol/water (3:1, v/v). After 40 min at 60°C, 1 ml of water and 1 ml of 6 N HCl were added to the mixture to acidify the phase (pH 3). Labeled compounds were extracted with chloroform/methanol (2:1, v/v) and rechromatographed using a solvent system of diethyl ether/hexane/acetic acid (70,30,1; v/v/v). The amount of radioactivity migrating with PL and free AA was determined by liquid scintillation counting.

In another set of experiments labeled PL were hydrolyzed with PLA_2 from bovine and porcine pancreas (Sigma). Briefly, labeled PL samples were resuspended in 1 ml of diethyl ether/methanol (95:5, v/v) containing 50 μl of Tris buffer (0.05 M), $CaCl_2$ (25 mM), EDTA (1 mM), and 1 mg of phospholipase. The reaction mixture was incubated at 37°C for 2 h with continuous shaking. After evaporation of solvent, samples were rechromatographed as for base hydrolysis.

2.6. Production of PGE_2, 6-Keto-$PGF_{1\alpha}$ and LTB_4 by human bone marrow stromal cells

Human bone marrow stromal cells (2×10^5) in 24-well plates were incubated in 1 ml of RPMI 1640 with $CaCl_2$ (2 mM) and $MgCl_2$ (0.5 mM) and stimulated with PMA (1 μM), IL-6 (10 ng/ml) and TNF-α (10 ng/ml) at 37°C for 5, 20, 60 and 180 min. Supernatants were recovered and stored at −80°C until assay. PGE_2, 6-keto-$PGF_{1\alpha}$ (the stable metabolite of PGI_2) and LTB_4 levels were determined with enzyme linked immunosorbent assay (ELISA) kits (Cayman Chemicals, Ann Harbor, MI). Results are reported as mean ± SEM of three independent experiments. Data were compared with Mann–Whitney U-test. A $P < 0.05$ was considered significant.

2.7. Cell proliferation

It was assessed by [^{3}H]-thymidine incorporation into DNA and cell counts as previously reported [13,14]. Briefly, cells (1×10^4 per well) were plated for 24 h in 96-well plates in 100 μl of culture medium. Adherent cells were washed with HBSS and 200 μl of serum-free medium was added to each well for 2 days. Adherent cells were reactivated with 100 μl of RPMI 1640 with 5% or 20% FCS. AA (Tebu, Le Perray-en-Yvelines, France) or the appropriate vehicle (10 μl of 2% HSA) were added immediately after reactivation. After 60 h of incubation, cultures were pulsed for 12 h with 1 μCi/ml [^{3}H]-thymidine (Amersham) and the cells were harvested using a Skatron cell harvester. Data are reported as mean dpm ± SEM of sixplicate samples. In separate sets of experiments, cells (in triplicate samples) were harvested after trypsin treatment (0.05% trypsin for 5 min at 37°C) and counted by using a haemocytometer. Results were compared with Student's *t*-test for paired samples. A $P < 0.05$ was considered significant.

3. Results

3.1. Fatty acid composition of human bone marrow plasma and marrow stromal cell cultures

The fatty acid compositions of the human bone marrow plasma and cultured marrow stromal cells are shown in Table 1. Enrichment of media with 1 μM AA significantly ($P < 0.05$, Mann–Whitney U-test, five experiments) increases this fatty acid in total marrow stromal cell lipids as compared with controls ($25.30 \pm 5.36\%$ vs. $9.52 \pm 0.40\%$ for AA-supplemented and control cells, respectively).

3.2. Incorporation of [^{3}H]-AA in bone marrow stromal cell lipids

After 10 min, 1, 5 and 24 h of incubation with [^{3}H]-AA, human bone marrow stromal cells had incorporated 10, 33, 75 and 76% of the initially added radioactivity, respectively (mean of three experiments). Lipids were extracted from labeled cells and separated into classes by TLC. The distribution of label in cell lipids as a function of time is shown in Fig. 1. A trafficking of labeled AA from TG to PL was observed. At 24 h, 83% of the label was recovered in PL while 10% was recovered in TG. Less than 5% of label was recovered in DG or as free AA. Labeled MG were not detected. The culture of cells for 48 h in serum-free medium before labeling or the presence of 20% FCS in the medium during labeling had no effect on the percentages of AA incorporated into PL, DG or TG (data not shown).

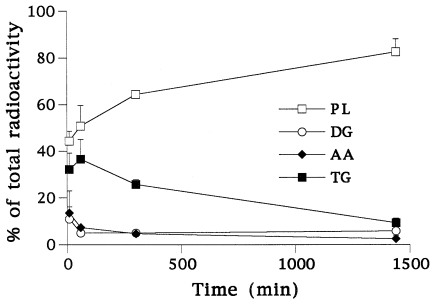

Fig. 1. Time-course of [^{3}H]-AA incorporation into human bone marrow stromal cell lipids. Cells were incubated with [^{3}H]-AA for 10 min, 1, 5, or 24 h. Lipids were extracted and separated as described in Section 2. Results are expressed as the percentage of total radioactivity found in cells. Mean ± SEM of three independent experiments in duplicate. PL: phospholipids; DG: diglycerides; TG: triglycerides; AA: arachidonic acid.

3.3. Incorporation of [^{3}H]-AA in bone marrow stromal cell PL species

We next investigated the distribution of [^{3}H]-AA into PL species using cells labeled for 24 h. The percentage (mean of three experiments) of incorporated AA into PE (mean 67%, range 60–71%) was greater than for PC (mean 18%, range 14–22%) and

Table 1
Fatty acid composition of human bone marrow plasma and human bone marrow stromal cell cultures

Major fatty acids	Marrow plasma ($n = 4$)	Marrow stromal cells ($n = 5$)
C14:0	1.70 ± 0.66	2.21 ± 0.26
C16:0	24.1 ± 0.86	25.25 ± 0.35
C16:1	3.33 ± 0.20	1.96 ± 0.70
C18:0	7.30 ± 0.30	21.11 ± 0.47
C18:1	35.54 ± 2.74	20.06 ± 0.89
C18:2	14.33 ± 2.33	4.10 ± 0.84
C20:4	2.5 ± 0.90	9.52 ± 0.40
Others[a]	11.20 ± 0.84	15.80 ± 1.34

Data (mean ± SEM) are reported as the percentage of fatty acid species.
[a]Others: includes the sum of all other fatty acids (not shown) that were $< 1.5\%$ of total fatty acids. C20:4 is arachidonic acid.

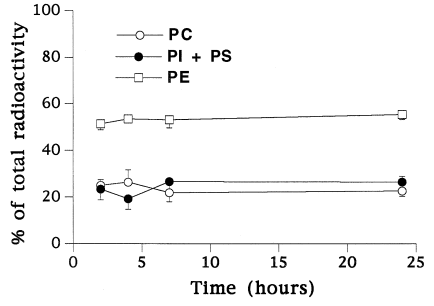

Fig. 2. Trafficking of [^{3}H]-AA into PL species. Cells were labeled for 2 h with [^{3}H]-AA. Medium was removed and cells were incubated with medium free of [^{3}H]-AA for 2, 5 or 22 h. Results are expressed as the percentage of total radioactivity found in PL species. Mean ± SEM of three independent experiments in duplicate. PC: phosphatidylcholine; PE: phosphatidylethanolamine; PI + PS: phosphatidylinositol + phosphatidyl serine (comigration).

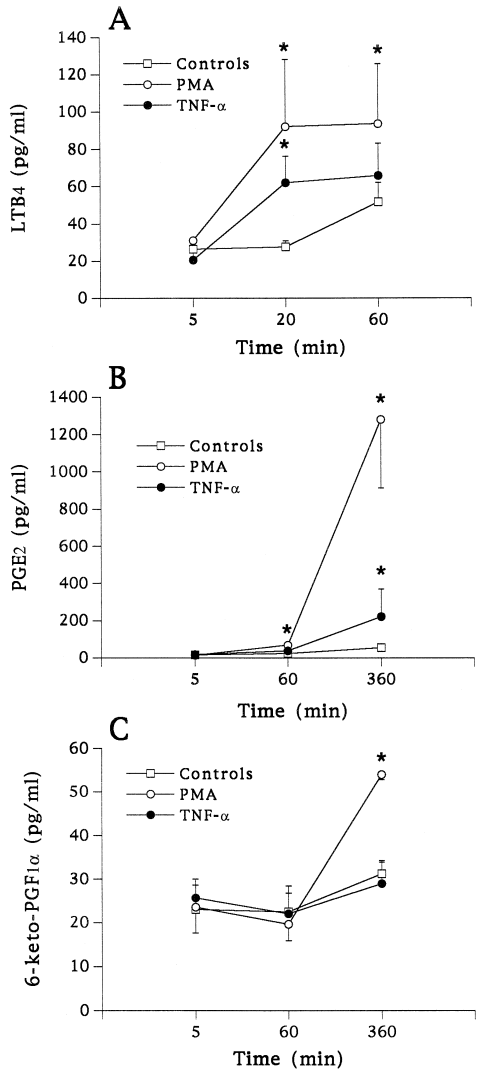

Fig. 3. Production of LTB_4 (A), PGE_2 (B) and 6-keto-$PGF_{1\alpha}$ (C) by human bone marrow stromal cells. Cells were stimulated with PMA (1 μM) and TNF-α (10 ng/ml) at 37°C for various periods of time. PGE_2, 6-keto-$PGF_{1\alpha}$ and LTB_4 levels were determined in cell supernatants by ELISA. Mean ± SEM of three independent experiments. $*$ $P < 0.05$ as compared to controls (Mann–Whitney U-test).

PI + PS (mean 14%, range 13–16%). No difference in the distribution of label was found when [^{3}H]-AA was incorporated with 20% FCS in the culture medium or using cells grown for 48 h in serum-free medium before labeling (data not shown). In a separate set of experiments we have investigated the putative exchange of labeled AA between PL species. Cells labeled for 2 h were washed and grown in culture medium free of [^{3}H]-AA for 2, 5 or 22 h. Under these experimental conditions no trafficking of [^{3}H]-AA was documented between the various PL species (Fig. 2).

Base hydrolysis of labeled PL resulted in the loss of 93% (mean of three experiments) of radioactivity, which was recovered as free fatty acid. Similarly treatment of labeled PL with PLA_2 from porcine pancreas or bovine pancreas lead to the loss of 92% and 91% (mean of two experiments) of label, respectively.

3.4. Production of PGE_2, 6-Keto-$PGF_{1\alpha}$ and LTB_4 by bone marrow stromal cells

As reported in Fig. 3, PMA (1 μM) and TNF-α (10 ng/ml) significantly ($P < 0.05$) stimulated PGE_2 and LTB_4 production by human bone marrow stromal cells. PMA significantly ($P < 0.05$) stimulated their 6-keto-$PGF_{1\alpha}$ synthesis. In our experimental conditions, IL-6 (10 ng/ml) was not a stimulant of PGE_2, 6-keto-$PGF_{1\alpha}$ and LTB_4 production by human bone marrow stromal cells (data not shown).

3.5. Effect of AA on bone marrow stromal cell growth

As reported in Table 2, the addition of AA (1 μM to 1 nM) had no significant ($P > 0.05$) effect on the [^{3}H]-thymidine incorporation by cells cultured in 5% or 20% FCS. No significant ($P = 0.17$, Mann–Whitney U-test, four experiments) effect of AA (1 μM) was found after 3 days of growth on the number of

Table 2
Effects of AA on [^{3}H]-thymidine incorporation of human bone marrow stromal cells

FCS	Controls	1 μM AA	100 nM AA	10 nM AA	1 nM AA
5%	2567 ± 469	2052 ± 335	2240 ± 407	2202 ± 405	2160 ± 365
20%	3947 ± 644	3988 ± 572	3563 ± 1022	3847 ± 773	4187 ± 1671

Cells were grown with 5% or 20% FCS in medium. Data (in dpm) are the mean ± SEM of six independent experiments in sixplicate.

viable cells ($55.4 \pm 8.2 \times 10^3$ cells) as compared with controls ($60.6 \pm 5.2 \times 10^3$ cells).

4. Discussion

Studies report the regulatory role of AA metabolites such as PGE_2 and LTB_4 in human bone marrow hematopoiesis [2–5]. Marrow stromal cells act on human hematopoiesis by secreting cytokines and growth factors that modulate the growth of bone marrow progenitors [6]. Marrow stromal cells also produce the lipidic mediator platelet-activating factor (PAF) [7] that enhances their growth [13]. In this study we have investigated the capacity of bone marrow stromal cells to capture AA, to produce some AA metabolites as well as the role of AA in their growth.

Using gas chromatography analysis, we report the presence of AA in human bone marrow plasma and marrow stromal cell cultures. Fatty acid cell composition is substantially modified by growing these cells in AA enriched media. Similarly to several other cell types [15–18], experiments with [^{3}H]-AA indicate that human bone marrow stromal cells incorporate AA into PL and TG. Time-course experiments indicate a transfer of AA from TG to PL. In some cell types the incorporation of AA into TG is considered to be an important mechanism to capture fatty acids in the culture medium for the biosynthesis of PL [19]. Base hydrolysis and PLA_2-treatment experiments demonstrate that AA is incorporated unmodified in PL and is linked by an ester bond. Prelabeling-chase experiments indicate a preferential incorporation of AA into PE and no trafficking of labeled AA between PL species.

While PAF stimulates the proliferation of human bone marrow stromal cells [13], AA has no effect on their growth assessed by [^{3}H]-thymidine incorporation into DNA and cell counts. Similarly, AA metabolites such as LTB_4, LTC_4, lipoxin A_4, lipoxin B_4, 12-HETE and 15-HETE have no effect on their growth [20].

We show, for the first time, that stimulated human bone marrow stromal cells produce some AA metabolites that act on the growth of human marrow erythroid and myeloid progenitors [2–5]. Thus, PMA is a stimulant of PGE_2, 6-keto-$PGF_{1\alpha}$ and LTB_4 production by human bone marrow stromal cells. TNF-α stimulates their PGE_2 and LTB_4 synthesis. In contrast, IL-6 has no effect on their PGE_2, 6-keto-$PGF_{1\alpha}$ and LTB_4 production. In this study PMA, IL-6 and TNF-α are used at concentrations that stimulate cytokine production by human bone marrow stromal cells. Thus, PMA stimulates their synthesis of macrophage colony stimulating factor (M-CSF), leukemia inhibitory factor (LIF), IL-6 and IL-8 [8,21–23]. TNF-α stimulates their LIF, IL-6 and IL-8 production [21,23] while IL-6 stimulates their LIF and IL-8 synthesis [21,23].

In conclusion human bone marrow stromal cells readily incorporate AA into their membrane PL and might participate in the control of marrow AA concentrations. PGE_2 and LTB_4 act on the growth of human marrow hematopoietic progenitors. Stimulated human bone marrow stromal cells produce PGE_2 and LTB_4 highlighting that they might modulate marrow hematopoiesis not only by producing cytokines but also by releasing lipid mediators.

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